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Research Article

Role of transcriptional factors Sp1, c-Rel, and c-Jun in LPS-induced C/EBPδ gene expression of mouse macrophages

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Abstract. Transcription factor C/EBPs are involved in the regulation of various cellular responses. Here, it was suggested that C/EBPδ gene was activated by lipopolysaccharide (LPS) through transcription factors Sp1, c-Rel, and c-Jun. Assay of the luciferase reporter vectors containing a 5′-deletion of the C/EBPδ gene promoter indicated that a LPS-responsive element was positioned between –345 and –35 bp of mouse C/EBPδ gene promoter. Transcription factors Sp1, c-Rel, and c-Jun bound to this region were identified using both *in vivo* chromatin immunoprecipitation and *in vitro* DNA-protein binding assays. LPS enhanced the proteins and DNA binding capacities of c-Rel and c-Jun, and the downstream Sp1 site was essential for LPS-induced C/EBPδ gene. Treatment of cells with ERK/JNK/p38 inhibitors or NF-κB inhibitor inhibited the LPS-induced C/EBPδ gene expression by inhibiting c-Jun, c-Rel, and p300 binding to DNA. Our findings provide a better understanding of LPS-induced C/EBPδ gene expression.

Keywords. Monocytes/macrophages, transcription factors C/EBPδ, Sp1, c-Rel, c-Jun, NF-κB, MAPK, LPS.

Introduction

Transcription factor CCAAT/enhancer-binding proteins (C/EBPs) are reported to be involved in the regulation of various aspects of cellular differentiation and inflammation [1]. There are at least six C/EBP family members (α, β, δ, γ, ε, ζ) that form homo- or heterodimers with C/EBPs or other leucine zipper proteins and bind to DNA to activate or repress transcription [2]. It is known that C/EBPβ and C/EBPδ play a synergistic role in terminal adipocyte differentiation *in vivo* [3] and are essential in LPS-induced cyclooxygenase-2 expression of mouse macrophages [4–6]. Different functions of these two C/EBP members were explored by gene knockout mice study [7, 8]. In C/EBPβ-deficient mice, defective immune responses including impaired bactericidal activity of macrophage and increasing susceptibility to infections were reported [7]. In a separate study of the C/EBPδ$^{-/-}$ mice, it was found that C/EBPδ played an important role in mammary epithelial cell growth control in the mouse nulliparous mammary gland [8], and the animals manifested a selectively enhanced

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contextual fear response in the contextual and auditory-cue-conditioned fear task of learning and memory [9]. Loss of C/EBPδ also causes genomic instability and centrosome amplifications in primary embryonic fibroblasts derived from 129S1 mice [10]. Recently, it was reported that C/EBPδ was essential for proteasome inhibitors-mediated cyclooxygenase-2 expression [11]. Acetylation of C/EBPδ was proven to be important for epidermal growth factor-induced cyclooxygenase-2 gene expression [12]. C/EBPδ protein is also increased in the pathologically vulnerable regions of the Alzheimer's disease brain, particularly in reactive astrocytes surrounding the amyloid β peptide deposits [13]. In AIDS-associated neurological disorders, HIV-induced increase in complement factor C3 is hypothesized to contribute to the neurodegeneration, where C/EBPδ is a key factor for HIV-induced C3 expression [14]. In the early stage of anti-Thy1.1 glomerulonephritis, C/EBPδ is induced and contributes to inflammatory gene expression and mesangial cells proliferation [15]. All the results support the notion that C/EBPδ is involved in growth/differentiation control and immunity/inflammation reactions.

The expression of C/EBPδ can be induced by a variety of stimuli including interleukin (IL)-6 [16], IL-1 [17], TNF-α [18], leukemia inhibitory factor/prostacyclin [19], epidermal growth factor [20], LPS [21], and peptidoglycan [22]. From the analysis of the promoter, it was found that IL-6 activated the C/EBPδ gene through acute-phase response factor/signal transducers and activators of transcription (APRF/STAT3) and Sp1 binding to its promoter in human hepatocytes [16, 23]. Stat3 also activates C/EBPδ transcription during G_0 growth arrest in mouse mammary epithelial cells [24]. Epidermal growth factor-induced C/EBPδ gene expression is mediated through p38 and CREB, which binds to the proximal promoter region of C/EBPδ gene in human epidermoid carcinoma cells [20]. C/EBPδ, as a transcription factor, also activates other gene after its induction by exogenous stimulation. For example, it activates gene expression of plasminogen activator inhibitor-1, which is an acute-phase reactant in vascular system [25], indicating that C/EBPδ is involved in regulation of acute-phase inflammation. We previously found that C/EBPδ was involved in LPS-induced gene expression of mouse IL-10 [26]. Our findings also illustrated that the expression of C/EBPδ protein was increased dramatically by LPS within a few hours, and overexpression of C/EBPδ activated the promoter activity of mouse IL-10 gene [26]. These results suggested that C/EBPδ is important in mediating immunological regulation by LPS. In an *in vivo* study, it was also reported that expression of C/EBPδ mRNA was enhanced in many tissues within 4 h following LPS treatment in mice [27].

Up to now, little has been known about the molecular mechanism of C/EBPδ gene activation induced by LPS. In this study, chromatin immunoprecipitation (ChIP) assay was employed to demonstrate that LPS induced recruitment of the transcription factors c-Rel and c-Jun to C/EBPδ promoter. Using a reporter gene assay and an *in vitro* DNA-protein binding assay, we found that Sp1, c-Rel, and c-Jun were the key proteins for LPS-induced C/EBPδ expression in mouse macrophages.

Materials and methods

Materials. LPS (*Salmonella typhosa*) and streptavidin-agarose beads were from Sigma (St. Louis, MO). The jetPEI™ was from Polyplus transfection (Illkirch cedex, France). The luciferase assay system, pGL2-Basic plasmid and U0126 were from Promega (Madison, WI). The endotoxin-free plasmid purification kit was from Qiagen (Hilden, Germany). The enhanced chemiluminescence kit was purchased from Amersham Biosciences (Buckinghamshire, UK). Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were obtained from Invitrogen Gibco (Carlsbad, CA). Antibodies against c-Rel, p50, p65, Stat3, Sp1 and C/EBPδ were from Santa Cruz (Santa Cruz, CA). Antibodies against c-Jun were from BD Transduction Labs (Lexington, KY). SB203580, SP600125 and Ro1069920 were from Tocris (Ballwin, MO). Biotinylated oligonucleotides were synthesized by MDBio Inc. (Taipei, Taiwan). All other reagents used were of the highest purity obtainable.

Cell culture and LPS treatment. Mouse macrophage RAW 264.7 cells were purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan) and cultured at 37 °C under 5 % CO_2 in a 75-cm^2 plastic flask containing 12 ml DMEM supplemented with 10 % FBS. In this series of experiments, cells were treated with 100 ng/ml LPS in culture medium supplemented with 10 % FBS.

Detection of mRNA by RT-PCR. Total RNA was isolated from RAW264.7 cells. Reverse transcription (RT) was performed on 2 μg total RNA by oligo(dT) primers and SuperScript™-II, then 1/20 volume of reaction mixture was pooled, followed by PCR with mouse C/EBPδ specific primers (5′-ATCGCTGCAGCTTCCTATGT-3′, 5′-GGTTAAGCCCGCAAACATTA-3′), mouse c-Rel specific primers (5′-GAGGGGAATGCGGTTTAGATACAA-3′, 5′-TGGGAGGCACAGCAGTTGTGAAGT-3′), mouse c-

Jun specific primers (5′-ACCGCGCGAGGAAATAGG-3′, 5′-CCGCTAGCACTCACGTTGGT-3′) or β-actin specific primers (5′-CCTAAGGCCAACCGTGAAAAG-3′, 5′-TCTTCATGGTGCTAGGAGCCA-3′). PCR was performed for 25 cycles. The PCR products were then analyzed by 1 % agarose gel.

Construction of luciferase reporter vectors. Mouse C/EBPδ promoter region (–1660/+25 bp) was prepared by PCR amplification of genomic DNA of RAW 264.7 cells with specific primers (5′-CCAAGTCATTTTGTTGCCACCC-3′ and 5′-GGGAAGCTTCCTGGCGTCCAAGTTGGCTG-3′). The DNA fragments were inserted into a luciferase plasmid pGL2-Basic to form plasmid δ1660. The DNA sequence was confirmed as that was reported by Cantwell et al. [16] and Hutt et.al. [24]. All the plasmids for transfection were purified using endotoxin-free plasmid purification kit.

Transfection of RAW 264.7 cells with jetPEI™. Cells were transfected with plasmids using jetPEI™ according to the manufacturer's instruction with slight modification. Cells were replated 16 h before transfection at a density of 2.8×10^5 cells in 1 ml of fresh culture medium in a 12-well plastic dish. For use in transfection, jetPEI™ was incubated with plasmids (1 μl jetPEI™/0.5 μg total plasmids) in 0.1 ml serum-free medium for 15 min at room temperature. Variable amounts of expression plasmids were compensated with the empty vector pcDNA3.1. 0.4 ml culture medium containing 10 % FBS was added to the DNA/jetPEI™ mixture. The mixture was added dropwise to the cells, and the cells were then incubated at 37 °C in a humidified atmosphere of 5 % CO_2 for 5 h. After changing the DNA/jetPEI™ medium to 1 ml of fresh culture medium, cells were treated with LPS for 5 h. The cell lysates were prepared for luciferase and protein concentration assays. The relative luciferase activities were normalized to the same protein concentration.

Nuclear extract preparation. Cells from 10-cm dishes were washed twice with PBS and scraped in 1 ml PBS. Cells were collected by centrifugation at 7500 *g* for 30 s, re-suspended in 0.4 ml buffer A (10 mM HEPES/pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$ and 0.5 mM EDTA) at 4 °C for 10 min. Nuclei were pelleted by centrifugation at 7500 *g* for 30 s. Pellets were resuspended in 0.1 ml buffer C (20 mM HEPES/pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 25 % glycerol) at 4 °C for 20 min. The suspension was centrifuged at 7500 *g* for 2 min. Supernatants were collected and stored at –80 °C until use. Both buffer A and C contain the following protease and phosphatase inhibitors: 0.5 mM phenylmethylsulfonyl, 1 mM orthovanadate, 2 μg/ml pepstatin A, and 2 μg/ml leupeptin.

ChIP assay. The assay was performed as described with a slight modification [28]. RAW 264.7 cells were treated with or without LPS for various times. Cells were cross-linked with 1 % formaldehyde at 37 °C for 15 min, washed twice with PBS, lysed with L1 buffer (50 mM Tris/pH 8.0, 2 mM EDTA, 0.1 % NP-40, 10 % glycerol), and then resuspended with L2 buffer (50 mM Tris/pH 8.0, 5 mM EDTA, 1 % SDS). The lysates were sonicated to shear the size of DNA to 500–1000 bp. Sonicated extracts were diluted tenfold with a dilution buffer (50 mM Tris/pH 8.0, 0.5 mM EDTA, 0.5 % NP-40, 1 M NaCl), followed by incubation with 50 μl salmon sperm DNA-saturated 50 % protein A-Sepharose at 4 °C for 2 h for pre-cleaning. Immunoprecipitation was performed with various antibodies with rotating at 4 °C overnight, followed by adding 40 μl salmon sperm DNA-saturated 50 % protein A-Sepharose at 4 °C for 1 h. Immunoprecipitation beads were pelleted and washed with high salt and low salt washing buffer each three times. DNA-protein complex was eluted in an elution buffer (1 × TE buffer containing 1 % SDS) with rotating at room temperature for 15 min, and the immune complex cross-link was reversed by heating at 65 °C for 2 h, followed by treatment with phenol/chloroform and precipitated with ethanol. The pellet was resolved in H_2O and subjected to PCR amplification using specific primers against C/EBδ promoter region: 5′-ACGGTTCACTAGTTCTGGTCTCG-3′ (C/EBPδ-329), and 5′-TTTTCTAGCCCCAGCTGACGC-3′ (C/EBPδ-24). The PCR products were separated by 1 % agarose-gel electrophoresis and visualized with ethidium bromide staining.

***In vitro* DNA-protein binding assay.** This assay was performed according to the previously reported method [29] with a slight modification. The binding assay was performed by mixing 150 μg nuclear extract proteins and 50 μl streptavidin-agarose beads with 20 % slurry at room temperature for 0.5 h for pre-cleaning. The supernatant was then incubated with 2 μg biotinylated C/EBPδ-specific oligonucleotide and 50 μl streptavidin-agarose beads with 20 % slurry. The mixture was incubated at room temperature for 1 h with rotating. Beads were pelleted and washed with cold PBS three times. The binding proteins were eluted by loading buffer and separated by SDS-PAGE, followed by Western blot analysis probed with specific antibodies.

Results

LPS-induced C/EBPδ gene expression in mouse macrophages. We first assessed the expression of C/EBPδ induced by LPS in mouse macrophages. RAW264.7 cells were incubated with 100 ng/ml LPS for various periods of time. LPS increased the C/EBPδ mRNA and protein expression level in a time-dependent manner. C/EBPδ mRNA induction was greatly increased after 2 h of treatment and was gradually decreased after 5 h of treatment (Fig. 1a). The increase in protein expression was observed after 3 h of treatment and was gradually decreased after 7 h of treatment (Fig. 1b). To assess whether the *de novo* protein synthesis was essential for LPS-induced C/EBPδ expression, cells were pretreated with cycloheximide for 1 h before LPS treatment. As shown in Figure 1c, treatment of cells with cycloheximide blocked the LPS-induced C/EBPδ mRNA, indicating that the *de novo* protein synthesis was essential for LPS-induced C/EBPδ gene expression.

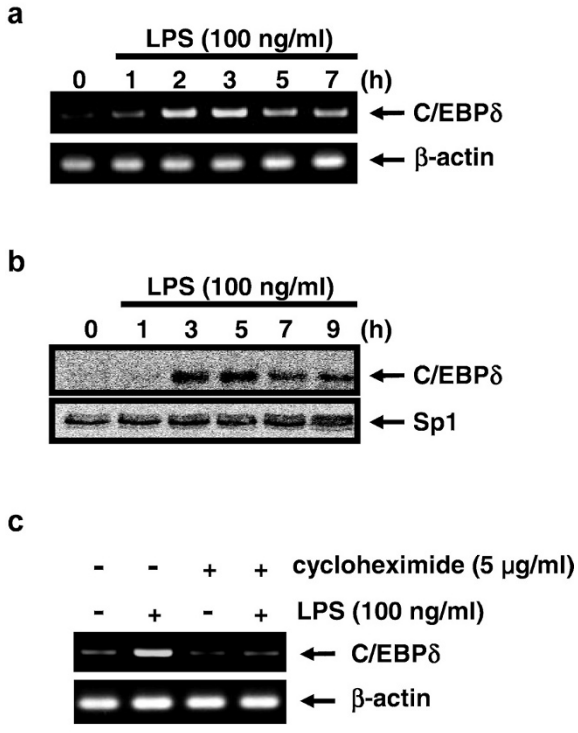

Figure 1. Time-dependent effect of LPS on C/EBPδ expression. Cells were stimulated with or without LPS (100 ng/ml) for various time. (*a*) Total RNA was extracted and subjected to RT-PCR using specific primers for C/EBPδ and β-actin (internal control). Products were analyzed by 1% agarose-ethidium bromide gel. (*b*) Nuclear extracts were extracted and subjected to Western blotting using C/EBPδ and Sp1 (as internal control) antibodies. (*c*) Cells were incubated for 1 h in the presence or absence of 5 μg/ml cycloheximide and then stimulated for 3 h with or without LPS (100 ng/ml) in the continuous presence or absence of the inhibitor. Total RNA was extracted and subjected to RT-PCR using specific primers for C/EBPδ and β-actin (internal control). Products were analyzed by 1% agarose-ethidium bromide gel.

Identification of the LPS-responsive element within the C/EBPδ gene promoter. To define the *cis*-acting elements necessary for LPS-induced C/EBPδ gene transcription, five different lengths of the mouse C/EBPδ promoter (Fig. 2a) were cloned into luciferase reporter vectors for the identification of the LPS-responsive element. Cells were transfected with these DNA constructs, and the inductions of LPS were studied. The basal luciferase activities of various lengths of C/EBPδ promoter are presented in Figure 2b. The basal activities were markedly decreased from δ101 to δ35, indicating that there were basal transcription factors binding to –101 to –35 bp of the C/EBPδ promoter. The LPS induction is summarized in Figure 2c, and the luciferase activity of the two different vectors with two different lengths of C/EBPδ promoter (δ1660, δ1045) was increased about eight- to tenfold after the LPS treatment. When the promoter sequence was deleted to –345 bp (δ345), –101 bp (δ101), or –35 bp (δ35), the LPS-induced luciferase activity was gradually decreased. These results indicated that there were LPS-responsive elements located at –1045 to –345, –345 to –101, and –101 to –35 bp of the C/EBPδ gene promoter. To simplify the study at the beginning, we first focused on studying the –345 to –35 bp region.

Identification of transcription factors bound to the C/EBPδ gene proximal promoter *in vivo*. Several potential transcription factor binding sites including Sp1, NF-κB, APRE and CRE motifs are present in the promoter of C/EBPδ ranging from –345 to –35 bp (Fig. 2a). The Sp1, APRE and CRE sites are important for C/EBPδ gene activation in different kinds of cell [16, 19, 20, 23]. In addition, there is a putative NF-κB binding site that is overlapped with the upstream Sp1 and APRE sites (Fig. 2a). Because each transcription factor binds to their specific DNA sequences and activates gene expression, we further identified which transcription factors play a role in the LPS-responsive region –345 to –35 bp of C/EBPδ gene promoter. We used a ChIP assay to identify the transcription factor binding to C/EBPδ proximal promoter *in vivo*. The primers C/EBPδ-329 and C/EBPδ-24 were used to specifically amplify the promoter region containing upstream Sp1 (–123 to –111 bp), NF-κB/APRE (–116 to –107/–110 to –102 bp), downstream Sp1 (–59 to –46 bp), and CRE (–45 to –34 bp) sites of the C/EBPδ gene locus by PCR (Fig. 3a). The predicted size of PCR product was correct as confirmed by agarose gel electrophoresis. As shown in Figure 3b, NF-κB p50, NF-κB p65, Sp1,

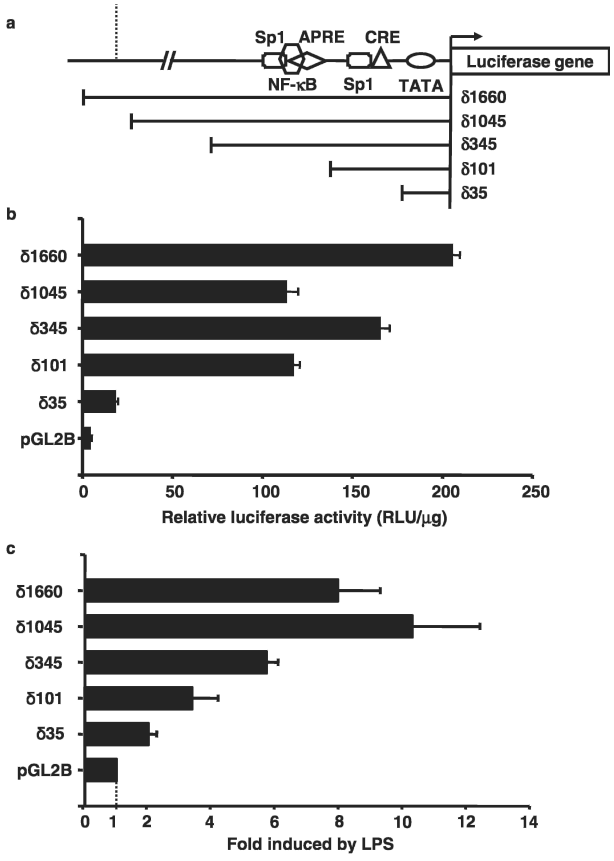

Figure 2. Transient expression of luciferase reporter gene vectors containing various lengths of C/EBPδ promoter. RAW264.7 cells were transfected with 0.5 μg luciferase reporter gene plasmid containing the C/EBPδ gene promoter. After changing the jet-PEI™/DNA medium to fresh culture medium, cells were stimulated, or not, with 100 ng/ml LPS for 5 h. The luciferase activity was normalized with protein concentration. (*a*) The 5′-deletion of C/EBPδ promoter fragments were ligated into luciferase reporter gene vector pGL2B as described in Materials and methods. (*b*) The luciferase activity of cells transfected with different 5′-deletion constructs. The relative luciferase activities were normalized to the same protein concentration. Values are means ± S.E.M. for three determinations. (*c*) LPS-induced luciferase activity of cells transfected with different 5′-deletion constructs. The fold induced by LPS is the ratio of LPS-treated to non-treated cells and corrected by pGL2B control vector (the LPS induction of pGL2B was set as 1 in each experiment). Values are means ± S.E.M. from more than three independent experiments.

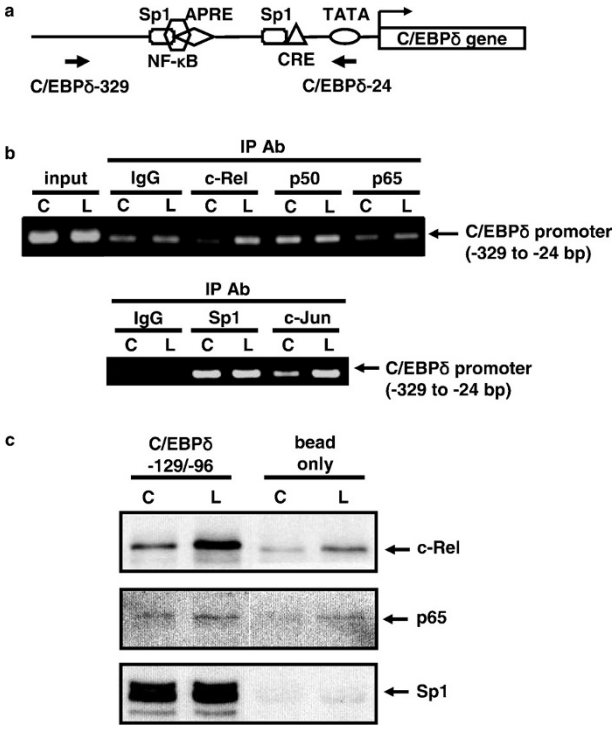

Figure 3. Analysis of specific proteins bound to C/EBPδ promoter *in vivo*. (*a*) Schematic representation of the C/EBPδ genomic locus spanning the proximal promoter region. Primer C/EBPδ-329 and primer C/EBPδ-24 were generated for PCR reaction. (*b*) ChIP analysis of c-Rel, p50, p65, Sp1 and c-Jun binding to the C/EBPδ gene locus. The chromatin was isolated from cells with (L) or without (C) LPS treatment for 2 h, sheared formaldehyde-cross-linked chromatin was immunoprecipitated with antibodies as indicated and processed for PCR amplification. The normal rabbit IgG is a background control in each experiment. As a positive control, PCR amplification also was carried out with input DNA from chromatin before the immunoprecipitation step. The PCR products were amplified with primers C/EBPδ –329 and C/EBPδ – 24 and then analyzed by 1 % agarose-ethidium bromide gel. (*c*) *In vitro* DNA-protein binding assay of nuclear extracts with sequence –129 to –96 bp of the C/EBPδ gene promoter. The oligonucleotides C/EBPδ –129 to –96 or bead only (background control) were incubated with nuclear extracts of RAW264.7 cells treated with (L) or without LPS (C) for 2 h as described in Materials and methods. The pellets were analyzed by Western blotting with antibodies against c-Rel, p65 and Sp1.

and c-Jun all bound to the promoter region of C/EBPδ gene in cells without LPS treatment. After stimulation with LPS, the binding of NF-κB c-Rel and c-Jun was dramatically increased, while that of NF-κB p50 and Sp1 remained unchanged. Although the binding of p65 was slightly increased by LPS in ChIP assay, the band signal was not higher than IgG control in three independent experiments; therefore, we further analyzed the binding of p65, c-Rel and Sp1 by *in vitro* DNA-protein binding assay. Like the ChIP assay result, the binding of c-Rel or Sp1 to C/EBPδ promoter –129/–96 bp was stronger than that of p65 (Fig. 3c). The c-Rel might therefore be more important than p65 in LPS-induced C/EBPδ expression. On the other hand, although Sp1 binding was not increased by LPS, this unchangeable Sp1 might also be important for gene activation like other genes [30, 31].

Identification of specific binding sites for c-Rel, c-Jun, and Sp1 in C/EBPδ gene promoter –345 to –35 bp. Because LPS enhanced the recruitment of c-Rel and c-Jun to C/EBPδ promoter *in vivo* (Fig. 3b) and LPS-induced C/EBPδ gene expression was mediated by *de novo* protein synthesis (Fig. 1c), we examined whether LPS induced protein synthesis of c-Rel and c-Jun. As shown in Figure 4a, LPS induced protein expression of

c-Rel and c-Jun in a time-dependent manner. In addition, LPS also caused an increased nuclear protein of c-Rel and c-Jun (Fig. 4b). Next, we further confirmed the binding sites of c-Rel, c-Jun, and Sp1 on LPS-activated C/EBPδ gene promoter by the *in vitro* DNA-protein binding assay with wild-type or point-mutated oligonucleotides. The two biotin-labeled double-stranded DNA probes corresponding to C/EBPδ promoter sequence were designed as shown in Figure 4c. Probe A contained −129 to −96 bp of C/EBPδ promoter including the potential binding sites of upstream Sp1 (−123 to −111 bp) and NF-κB/APRE (−116 to −107/−110 to −102 bp) sites, while Probe B contained −64 to −31 bp including downstream Sp1 (−59 to −46 bp) and CRE (−45 to −34 bp) sites. As shown in Figure 4d, Sp1 and NF-κB c-Rel bound to −129 to −96 bp of wild-type C/EBPδ promoter, and only c-Rel binding was increased after LPS stimulation. The Sp1 binding activity was eliminated in the Sp1-site mutated probe, c-Rel binding was decreased in the NF-κB/APRE-site mutated probe, and both Sp1 and c-Rel were decreased in the Sp1/NF-κB/APRE-double mutated probe (Fig. 4d). The results indicated that Sp1 and c-Rel directly bound to Sp1 (−123 to −111 bp) and NF-κB/APRE (−129 to −96 bp) sites of C/EBPδ promoter, respectively, where c-Rel binding was increased after LPS treatment. In the downstream fragment, using this streptavidin-agarose pulldown assay, we also detected that both Sp1 and c-Jun bound to −64 to −31 bp of C/EBPδ promoter, and the binding activity of c-Jun was increased after LPS treatment (Fig. 4e). Although c-Jun binding was decreased in CRE-mutated probe, the binding of c-Jun and Sp1 were both eliminated in Sp1–2-single mutated or Sp1–2/CRE-double mutated probes. Therefore, the downstream Sp1 site may be more important than the CRE site for both Sp1 and c-Jun binding.

Functional analysis of transcription factor binding sites in LPS-activated C/EBPδ gene promoter by point-mutated reporter gene assay. We next examined the promoter-driven activity by point-mutated reporter gene assay system. As shown in Figure 5a, various mutated constructs with one- to four-site mutations were constructed with luciferase gene. The basal luciferase activities of all mutant constructs were lower than that of wild type (δ345) (Fig. 5b). This indicated that these sites were functional in the basal expression of CEBPδ. In LPS induction, the induction folds of single mutation on upstream Sp1 (δ345mSp1) or NF-κB/APRE (δ345mNA) were not decreased comparing to the wild type (δ345), but were partially decreased when these two sites were mutated together (δ345mSp1/NA) (Fig. 5c). Because these two sites were overlapping and continuous, it was suggested that this sequential sequence (–123 to –102 bp) played a partial role in LPS-induced C/EBPδ expression. In contrast to upstream Sp1 site, the induction of LPS was largely decreased in downstream Sp1-single mutation (δ345mSp1–2). Although the induction was increased in single CRE-site mutation (δ345mCRE), it was still decreased in δ345mSp1–2/CRE. This suggested that downstream Sp1 was very important for LPS-induced C/EBPδ expression. In comparison to the δ345mSp1–2, the LPS induction was decreased a little more in four-site mutations (δ345 m2Sp1/NA/CRE) (Fig. 5c). Thus, the downstream Sp1 site may be the key sequence and upstream Sp1/NF-κB/APRE site a minor sequence for LPS-induced C/EBPδ promoter activity.

Effect of overexpression of Sp1, c-Jun or c-Rel in C/EBPδ promoter activity. To examine the functional roles of Sp1, c-Rel, and c-Jun in the promoter activity of C/EBPδ, we transiently co-transfected cells with δ345 and various expression vectors including Sp1, c-Jun, and c-Rel, and the luciferase activities were monitored. As shown in Figure 6a, Sp1 expression vector increased the luciferase activity of δ345 in a dose-dependent manner. At a dose between 0.125 and 0.0635 μg, c-Jun also increased the luciferase activity of δ345 in a dose-dependent manner, but a slightly decreased fold was presented at a dose of 0.25 μg. Overexpression of c-Rel at doses of 0.5 and 0.25 μg also significantly increased the luciferase activity of δ345. To investigate the additive effect of combinations of these transcription factors, we overexpressed two- or three-combination of Sp1, c-Jun, and c-Rel in cells. As shown in Figure 6b, the reporter gene expression almost reached the maximum response when Sp1 and c-Jun transcription factors were simultaneously overexpressed, and a little more increase when c-Rel was added. This suggests that all of transcription factors Sp1, c-Rel, and c-Jun activated C/EBPδ gene expression in mouse macrophages. The effect of overexpressed proteins on δ345 and mutants were also analyzed. Compared to δ345, δ345mCRE was activated by c-Jun overexpression (Fig. 7a), δ345mNA was activated by c-Rel overexpression (Fig. 7b), and δ345mSp1 and δ345mSp1–2 were activated by Sp1 overexpression (Fig. 7c). It was possible that these transcription factors might activate promoter through protein-protein interaction of other DNA binding proteins. Therefore, we also compared δ345 m2Sp1/NA/CRE activation by c-Jun, c-Rel, or Sp1 overexpression. The effect of c-Jun, c-Rel and Sp1 on δ345 m2Sp1/NA/CRE activity was decreased (Fig. 7d) compared to their effects on δ345. Therefore, all these binding sites might be influenced by each

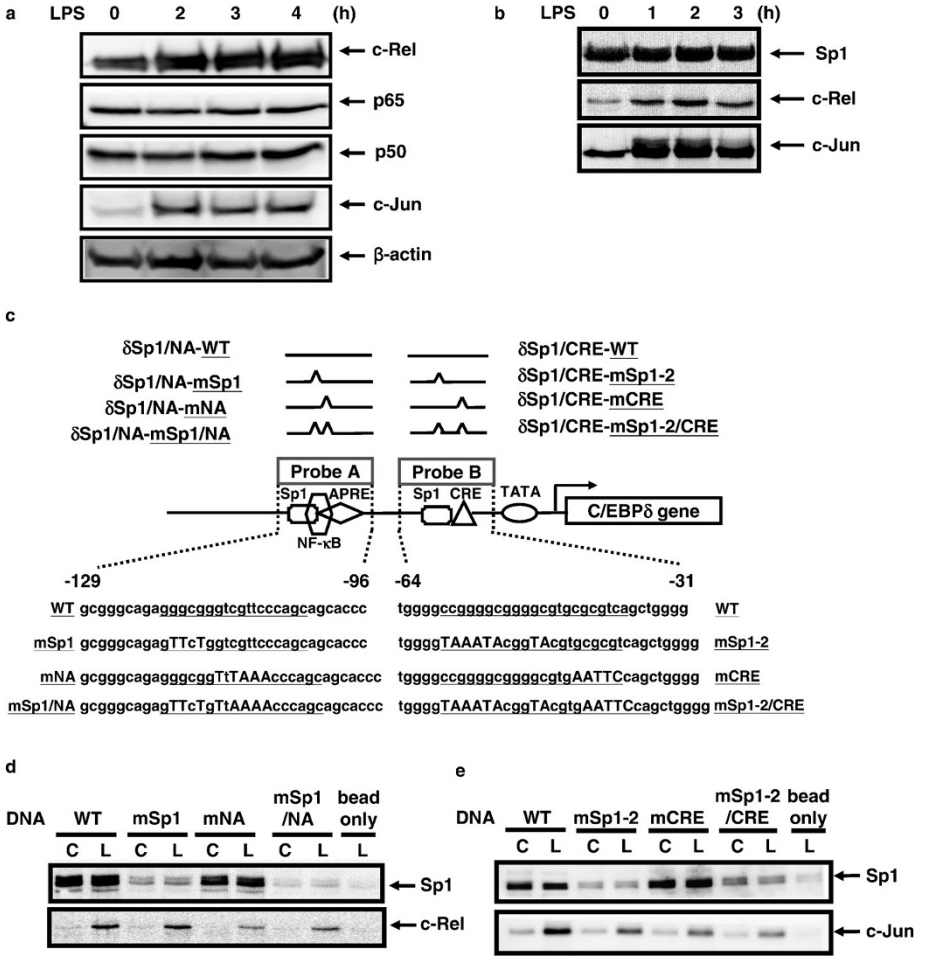

Figure 4. *In vitro* DNA-protein binding assay of nuclear extracts with sequence –129 to –96 bp (Probe A) and –64 to –31 bp (Probe B) of the C/EBPδ gene promoter. (*a*) The effect of LPS on total cellular protein expression of c-Rel, p50, p65, and c-Jun. Cells were treated with 100 ng/ml LPS for 0, 2, 3, and 4 h, then total cell lysates were collected for Western blotting using c-Rel, p65, p50, c-Jun and β-actin (as internal control) antibodies. (*b*) Time course of LPS-induced nuclear c-Rel and c-Jun proteins expression. Cells were treated with LPS for various time, and cell nuclear extracts were isolated for Western blotting. Specific antibodies of Sp1, c-Rel, and c-Jun were used for detecting their antigens as indicated. (*c*) The DNA sequences of wild-type and various point-mutated oligonucleotides covering the C/EBPδ promoter region from –129 to –96 bp (Probe A) and –64 to –31 bp (Probe B). (*d*) DNA-protein binding assay of wild-type and point-mutated –129 to –96 bp oligonucleotides (Probe A). The wild-type and point-mutated oligonucleotides were incubated with nuclear extracts of RAW264.7 cells treated with (L) or without LPS (C) for 2 h as described in Materials and methods. The pellets were analyzed by Western blotting with antibodies against Sp1 and c-Rel. (*e*) DNA-protein binding assay of wild-type and point-mutated –64 to –31 bp oligonucleotides (Probe B). The wild-type and point-mutated oligonucleotides were incubated with nuclear extracts of RAW264.7 cells treated with (L) or without LPS (C) for 2 h as described in Materials and methods. The pellets were analyzed by Western blotting with antibodies against Sp1 and c-Jun. Bead only means a background control; there is no DNA oligonucleotide in the mixture reaction.

other. When all of them were mutated, the induction of LPS or relative transcription factors was down to the lowest level. In addition, to identify the minimal stimulation of c-Rel on δ345, we compared its effect on δ345 to δ1045. As shown in Figure 7e, the degree of activation induced by c-Rel was similar between δ345 (2.1-fold) and δ1045 (2.0-fold), suggesting that δ345 was enough for c-Rel activation.

Involvement of MAPKs, NF-κB, and p300 in LPS-induced C/EBPδ gene expression. In our previous study, three MAPK inhibitors (U0126, SP600125, and SB203580) or NF-κB inhibitor (Ro106–9920) inhibited LPS-induced C/EBPδ protein expression individually [32], they also inhibited LPS-induced C/EBPδ mRNA expression (Fig. 8a). Since the *de novo* synthetic c-Rel and c-Jun were two of upstream mediators in LPS-induced C/EBPδ gene, we next examined whether the mRNA and protein expressions of c-Rel and c-Jun were influenced by these inhibitors. As shown in Figure 8b, LPS also induced mRNA expression of c-Rel and c-Jun. On pre-treatment with inhibitors, LPS-induced c-Rel mRNA was mainly decreased by NF-κB inhibitor and partially decreased by three MAPK inhibitors (Fig. 8c). In contrast, LPS-induced c-Jun mRNA was totally inhibited by three

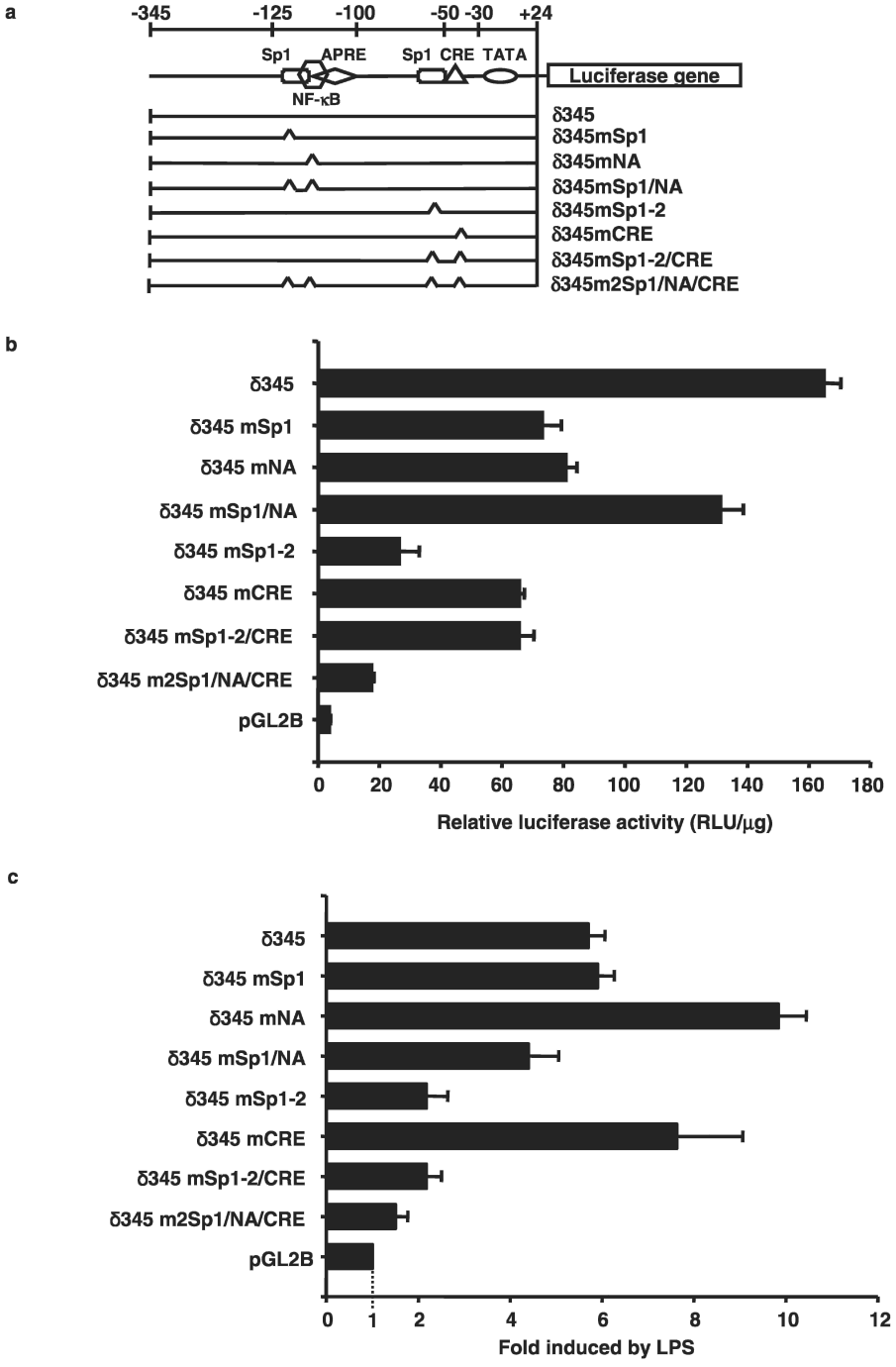

Figure 5. Transient expression of luciferase reporter gene vectors containing various site-directed mutants of C/EBPδ promoter. RAW264.7 cells were transfected with 0.5 μg luciferase reporter gene plasmid containing the C/EBPδ gene promoter. After changing the jetPEI™/DNA medium to fresh culture medium, cells were stimulated with 100 ng/ml LPS for 5 h or not. The luciferase activity was normalized with protein concentration. (*a*) The site-directed mutants of C/EBPδ promoter fragments were ligated into luciferase reporter gene vector pGL2B as described in Materials and methods. (*b*) The luciferase activity of cells transfected with wild type or site-directed mutation constructs. The relative luciferase activities were normalized to the same protein concentration. Values are means ± S.E.M. for three determinations. (*c*) LPS-induced luciferase activity of cells transfected with wild type or site-directed mutation constructs. The fold increase induced by LPS is the ratio of LPS-treated to non-treated cells and corrected by pGL2B control vector (the LPS induction of pGL2B was set as 1 in each experiment). Values are means ± S.E.M. from more than three independent experiments.

MAPK inhibitors and partially decreased by NF-κB inhibitor (Fig. 8d). The inhibitory phenomenon was also observed at the protein levels for c-Rel and c-Jun (Fig. 8e). In the *in vitro* DNA-protein binding assay, the binding ability of c-Rel to Probe A-WT (−129 to −96 bp) was eliminated by NF-κB inhibitor, and that of c-Jun to Probe B-WT (−64 to −31 bp) was decreased by the combination of three MAPK inhibitors (Fig. 8f). Therefore, the LPS-induced c-Rel binding to C/EBPδ promoter may be mainly mediated by NF-κB pathway, and LPS-induced c-Jun binding to C/EBPδ promoter mainly by MAPK pathways. Because either MAPK or NF-κB pathway inhibitors could inhibit LPS-induced C/EBPδ gene expression, we speculated that there was another factor that was inhibited by any one of these two pathways. Because CBP/p300 functions as a coactivator for many transcription activators [33], and its quantity was not changed by LPS and inhibitors (Fig. 8e), we analyzed its binding capacity for the C/EBPδ promoter. As

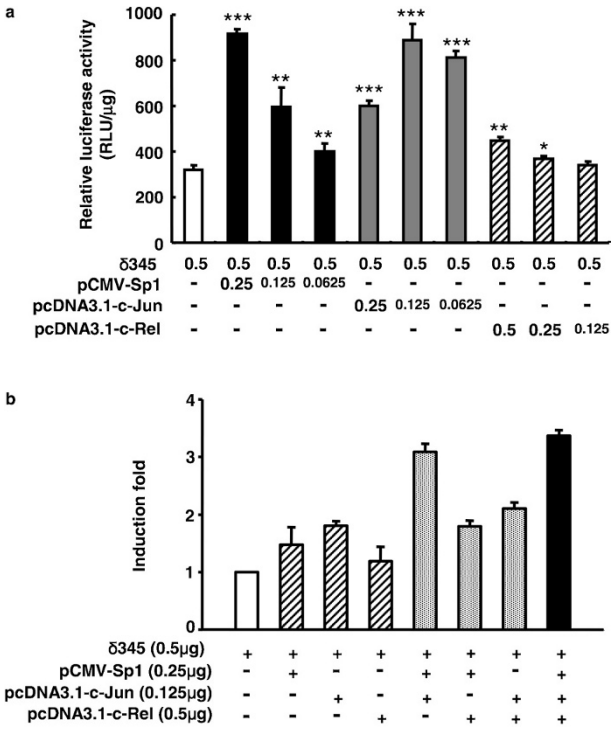

Figure 6. The effect of overexpressed Sp1, c-Jun, and c-Rel on the promoter activity of C/EBPδ gene. (*a*) Dose-dependent effect of overexpressed proteins on the promoter activities of C/EBPδ gene. RAW264.7 cells were transfected with 0.5 μg δ345 and various overexpression plasmids. After changing the jetPEI™/DNA medium to fresh culture medium, cells were incubated for another 5 h. The luciferase activity was normalized with protein concentration. Values are means ± S.E.M. of triplicates for each group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. δ345 only. (*b*) The effect of various combinations of overexpressed proteins on the promoter activity of C/EBPδ gene. RAW264.7 cells were transfected with 0.5 μg δ345 and various combinations of overexpression plasmids. After changing the jetPEI™/DNA medium to fresh culture medium, cells were incubated for another 5 h. The luciferase activity was normalized with protein concentration. The induction fold is the ratio of each group to δ345 only group (the induction fold of the δ345 only group was set as 1 in each experimental). Values are means ± S.E.M. of three independent experiments.

shown in Figure 8f, the binding of p300 to Probe B-WT (−64 to −31 bp) was increased by LPS and decreased by the three MAPK inhibitors or NF-κB inhibitor individually. Taken together, our results suggested that LPS-induced C/EBPδ gene expression was mediated through the signaling pathways of MAPK to c-Jun, NF-κB to c-Rel, MAPK to p300, and NF-κB to p300 pathways.

Discussion

In the present study, we found that the transcription factors Sp1, c-Rel, and c-Jun were all involved in the LPS-induced C/EBPδ expression in mouse macrophages, and the signaling pathways involved NF-κB, ERK, JNK and p38. Initially, the most important clue was the requirement of the *de novo* protein synthesis for LPS-induced C/EBPδ mRNA expression (Fig. 1c). Next, in a ChIP assay, it was shown that Sp1, c-Rel and c-Jun bound to C/EBPδ promoter region −329 bp to −24 bp in cells, and the bindings of c-Rel and c-Jun were increased after LPS treatment (Fig. 3b). From the point-mutated reporter gene assay (Fig. 5c), it appears that the downstream Sp1 site was essential for LPS-induced C/EBPδ expression. Taken together, these results suggest that Sp1 and the *de novo* synthesis of c-Jun and c-Rel are the key factors in LPS-induced C/EBPδ gene.

As shown in Figure 4c, all mutated sequences were analyzed by Program Patch 1.0-public on Gene Regulation website (www.gene-regulation.com). The analytic results showed that each mutated sequence completely lost the binding activity of NF-κB, Sp1, or c-Jun. However, the *in vitro* DNA-protein binding assay showed that there was some remaining c-Rel binding with the mNA oligonucleotide, some c-Jun binding with the mCRE oligonucleotide, and some Sp1 binding with the mSp1 or mSp1–2 oligonucleotides (Fig. 4d, e). This phenomenon might be due to the inconsistence between computer and biochemical analyses, or the remaining bindings were from protein-protein interaction of other unknown DNA binding proteins. In spite of incomplete elimination, all the designed mutation could significantly attenuate the binding ability of c-Rel, c-Jun and Sp1 respectively.

Our results indicated that downstream Sp1 site was critical for LPS-induced C/EBPδ gene (Fig. 5c). The results of the *in vitro* DNA-protein binding assay indicated that this downstream Sp1 site provides the binding site for Sp1 and c-Jun (Fig. 4e). According to the sequence of the downstream Sp1 site, this serves the direct binding site for Sp1, not c-Jun. However, it is known that Sp1 can recruit other transcription factors, *e.g.*, c-Jun [34, 35], YY1 [36], NF-κB p65 [37] and C/EBPδ [38], to activate gene expression. In studying the interaction between c-Jun and Sp1, it was reported that the C-terminus of c-Jun is the direct binding domain for Sp1 as shown by *in vitro* binding assay [39]. In the present study, we found that c-Jun binds to CRE site of C/EBPδ promoter and also binds to Sp1 site (Fig. 4e), which might be mediated *via* the Sp1 interaction. Sp1 belongs to a zinc finger family of transcription factors that recognizes the GC-rich DNA sequence [40]. In the LPS-stimulated RAW 264.7 cells, the quantity and DNA binding capacity of nuclear Sp1 were not changed, and it is possible that transcriptional activity of Sp1 is enhanced by LPS through c-Jun and p300 recruitment (Fig. 7f), or by post-translational modification of Sp1, *e.g.*, deacetylation [41]. Because p300 is a critical coactivator for acetylating histone and activat-

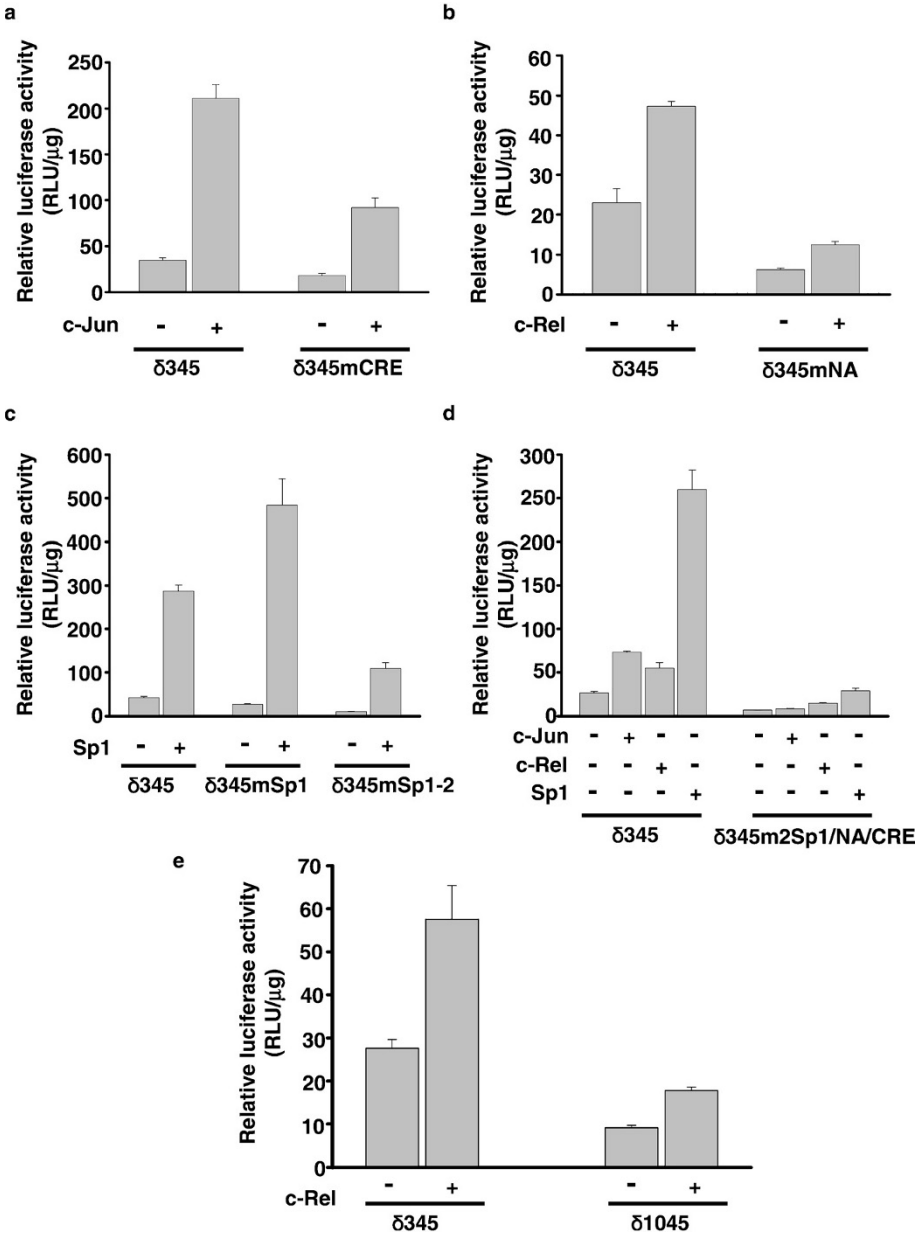

Figure 7. The effect of overexpressed Sp1, c-Jun, and c-Rel on the promoter activity of wile-type and various mutants. (*a*) The effect of overexpressed c-Jun on the promoter activity of δ345 and δ345mCRE. RAW264.7 cells were transfected with 0.5 μg δ345 (or δ345mCRE) and 0.125 μg pcDNA3.1-c-Jun. After changing the jetPEI™/DNA medium to fresh culture medium, cells were incubated for another 5 h. The luciferase activity was normalized with protein concentration. Values are means ± S.E.M. of triplicates for each group. (*b*) The effect of overexpressed c-Rel on the promoter activity of δ345 and δ345mNA. RAW264.7 cells were transfected with 0.5 μg δ345 (or δ345mNA) and 0.5 μg pcDNA3.1-c-Rel. After changing the jetPEI™/DNA medium to fresh culture medium, cells were incubated for another 5 h. The luciferase activity was normalized with protein concentration. Values are means ± S.E.M. of triplicates for each group. (*c*) The effect of overexpressed Sp1 on the promoter activity of δ345, δ345mSp1, and δ345mSp1–2. RAW264.7 cells were transfected with 0.5 μg δ345 (or δ345mSp1 or δ345mSp1–2) and 0.25 μg pCMV-Sp1. After changing the jetPEI™/DNA medium to fresh culture medium, cells were incubated for another 5 h. The luciferase activity was normalized with protein concentration. Values are means ± S.E.M. of triplicates for each group. (*d*) The effect of overexpressed c-Jun, c-Rel or Sp1 on the promoter activity of δ345 and δ345 m2Sp1/NA/CRE. RAW264.7 cells were transfected with 0.5 μg δ345 (or δ345 m2Sp1/NA/CRE) and 0.125 μg pcDNA3.1-c-Jun, 0.5 μg pcDNA3.1-c-Rel, or 0.25 μg pCMV-Sp1. After changing the jetPEI™/DNA medium to fresh culture medium, cells were incubated for another 5 h. The luciferase activity was normalized with protein concentration. Values are means ± S.E.M. of triplicates for each group. (*e*) The effect of overexpressed c-Rel on the luciferase activity of δ345 and δ1045. RAW264.7 cells were transfected with 0.5 μg δ345 (or δ1045) and 0.5 μg pcDNA3.1-c-Rel. After changing the jetPEI™/DNA medium to fresh culture medium, cells were incubated for another 5 h. The luciferase activity was normalized with protein concentration. Values are means ± S.E.M. of triplicates for each group.

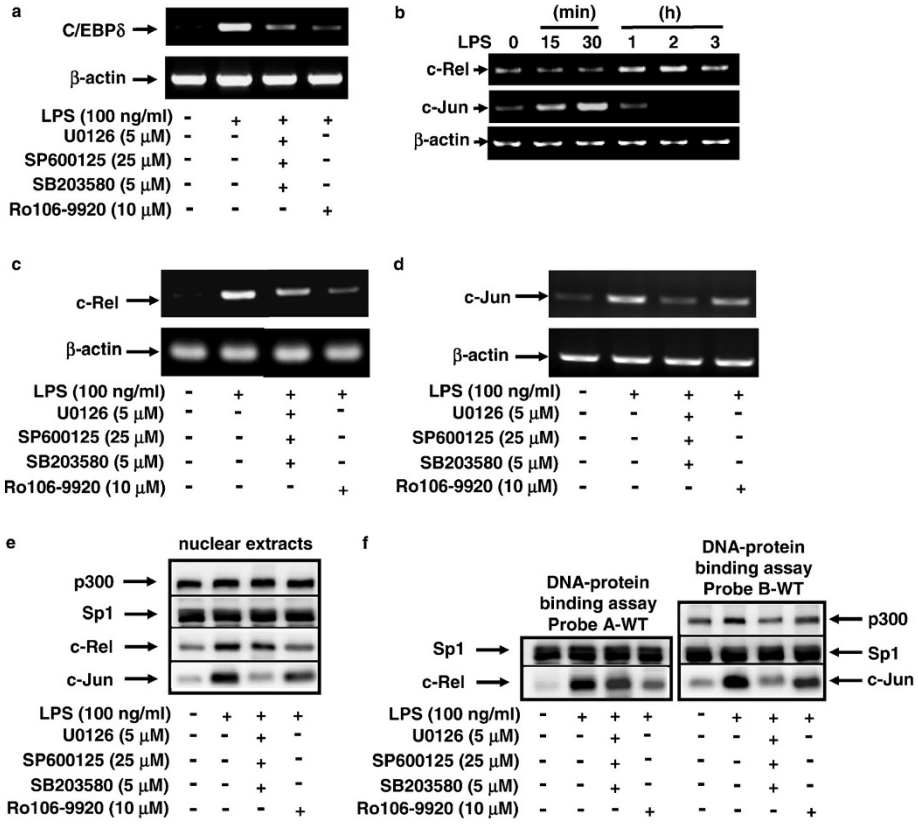

Figure 8. The effect of LPS and signal protein inhibitors on the expression of C/EBPδ and related transcription factors. (*a*) The effect of inhibitors on LPS-induced C/EBPδ mRNA. Cells were treated with various inhibitors, as indicated, 1 h before the 2-h LPS treatment. The total RNA was isolated, and C/EBPδ mRNA was detected by RT-PCR. (*b*) Time course of LPS-induced c-Rel and c-Jun mRNA expression. Cells were treated LPS for various time, and total RNA was isolated. The mRNA of c-Rel and c-Jun were detected by RT-PCR. (*c*) The effect of inhibitors on LPS-induced c-Rel mRNA. Cells were treated with various inhibitors, as indicated, 1 h before the 2-h LPS treatment. Total RNA was isolated and c-Rel mRNA was detected by RT-PCR. (*d*) The effect of inhibitors on LPS-induced c-Jun mRNA. Cells were treated with various inhibitors, as indicated, 1 h before the 30-min LPS treatment. Total RNA was isolated and c-Jun mRNA was detected by RT-PCR. (*e*) The effect of inhibitors on LPS-stimulated nuclear p300, Sp1, c-Rel, and c-Jun expression. Cells were treated with various inhibitors, as indicated, 1 h before the 2-h LPS treatment. Cell nuclear extracts were isolated for Western blotting with antibodies against p300, Sp1, c-Rel, and c-Jun. (*f*) The effect of inhibitors on DNA-protein binding of –129 to –96 bp (Probe A) and –64 to –31 bp (Probe B) of the C/EBPδ gene promoter. The cell nuclear extracts from (*e*) were applied for *in vitro* DNA-protein binding assay. The DNA-protein binding pellets were analyzed by Western blotting with antibodies against p300, Sp1, c-Rel, or c-Jun.

ing many genes [33] including C/EBPδ [42], loss of p300 binding by MAPKs inhibitors or NF-κB inhibitor (Fig. 8f) would inhibit C/EBPδ gene activation (Fig. 8a). Compared to the essential factor Stat3 in IL-6-induced and growth arrest-induced C/EBPδ gene activation [16, 24], Stat3 appears to be not important in LPS-induced C/EBPδ gene expression since the DNA binding of Stat3 did not change in ChIP assay (data not shown). Of course, we do not exclude other unknown factors, which are also important for LPS-induced C/EBPδ gene expression.

As indicated in Figure 5c, the LPS-induced effect of single mutation in NF-κB/APRE (δ345mNA) or CRE (δ345mCRE) was increased more than that of the wild type (δ345), but double mutation with upstream Sp1 and NF-κB/APRE (δ345mSp1/NA), or downstream Sp1 and CRE (δ345mSp1–2/CRE) was decreased more than that of the wild type. Because the NF-κB/APRE-site and CRE-site are near an individual Sp1 site, their mutations might strengthen the transcriptional activity of Sp1 because there is more space for other proteins interacting with Sp1. Therefore, the Sp1 site may be more important than NF-κB/APRE-site and CRE-site in LPS-induced C/EBPδ expression.

Akin to Sp1, c-Jun, and p300, NF-κB c-Rel also plays a role in LPS-induced C/EBPδ gene. It bound to the NF-κB/APRE binding site and activated C/EBPδ gene (Figs. 3b and 6a). Transcription factor c-Rel is one member of NF-κB family, and unlike the universally expressed p50 and p65 in most cell type, it is found predominantly in hematopoietic cells [43]. In this study, c-Rel total protein, nuclear protein and mRNA were increased upon LPS stimulation (Figs. 4a, b and 8b). Its binding to the promoter of C/EBPδ gene was also increased by the measurement in *in vivo* (Fig. 3b) and *in vitro* (Fig. 4d) assays. In macrophages, c-Rel is

more potent than p65 in the induction of IL-12 p40 gene [44], while it also increases IL-12 p35 gene expression in CD8(+) dendritic cells [45]. The location of c-Rel is also important for targeting the gene expression, for example, defective translocation of c-Rel is associated with impaired Th1 cytokine production in T cells from atopic dermatitis patients [46]. Calmodulin binds to c-Rel released from I kappa B, and thus inhibits nuclear import of c-Rel and prevents c-Rel acting on its target genes [47]. All these results suggest that not only p65 and p50, but also c-Rel is an important member of NF-κB for activating gene expression, and our present findings indicated that c-Rel was one of the factors in LPS-induced C/EBPδ gene expression. The necessity of the NF-κB/IKK pathway for C/EBPδ gene expression has also been reported by others [48].

In the study of signal pathways, LPS-induced mRNA expression of C/EBPδ and c-Jun were partially inhibited by only one of three MAPK inhibitors (data not shown), but they were completely inhibited by combination of three MAPK inhibitors (Fig. 8a, d). Because the inhibition of the three MAPK inhibitors combined was superior to the single inhibitor, we used a combination of the MAPK inhibitors for further study. On the other hand, only the NF-κB inhibitor inhibited LPS-induced mRNA expression of C/EBPδ and c-Rel (Fig. 8a, c). Because c-Jun and c-Rel are two of the important factors for LPS-induced C/EBPδ mRNA expression, inhibiting the protein expression of c-Jun and c-Rel and their DNA binding activities by these inhibitors consequently inhibited C/EBPδ gene expression. Furthermore, LPS-induced p300 binding to Probe B also was blocked by these inhibitors (Fig. 8f), but it is still unclear how these inhibitors inhibited p300 binding to C/EBPδ promoter.

In conclusion, LPS-induced C/EBPδ protein expression was mediated through ERK/JNK/p38 and NF-κB pathways, followed by the enhancement of the protein expression of their downstream transcription factors c-Jun and c-Rel. Not only was the increase of transcription factors c-Jun and c-Rel important for LPS-induced C/EBPδ gene expression, Sp1 protein, which was not increased after LPS treatment, was also essential for LPS-induced C/EBPδ gene expression in mouse macrophages. Because C/EBPδ is a critical gene for pathological inflammation, the mechanism of C/EBPδ gene activation by LPS as illustrated here provides a clue for a better understanding of LPS-induced inflammatory gene activation.

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